The brain of the Remipedia (Crustacea) and an alternative hypothesis on their phylogenetic relationships

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Remipedia are rare and ancient mandibulate arthropods inhabiting almost inaccessible submerged cave systems. Their phylogenetic position is still enigmatic and the subject of extremely controversial debates. To contribute arguments to this discussion, we analyzed the brain of *Godzilliognomus frondosus* Yager, 1989 (Remipedia, Godzilliidae) and provide a detailed 3D reconstruction of its anatomy. This reconstruction yielded the surprising finding that in comparison with the brain of other crustaceans such as representatives of the Branchiopoda and Maxillopoda the brain of *G. frondosus* is highly organized and well differentiated. It is matched in complexity only by the brain of ''higher'' crustaceans (Malacostraca) and Hexapoda. A phylogenetic analysis limited to brain anatomy across the Mandibulata strongly contradicts the prevailing hypothesis that the Remipedia are a basal, ancestral crustacean group but instead argues in favor of a remipede–malacostracan–hexapod clade and most likely a sister-group relationship of Remipedia and Malacostraca.

The discovery of the Remipedia in 1979 (1) was among the most important findings in crustacean biology in the second half of the 20th century. All 12 remipede species known so far live in cryptic submerged cave systems, often in karst coastal settings and typically with inland surface openings and subsurface connections to the nearby ocean (2–4). These animals mostly occur below the density interface between the lower seawater and the overlying well oxygenated freshwater lens. Remipedes lack any kind of eyes because their habitat is absolutely aphotic, and chemical (and tactile) clues most likely play a major role for orientation.

The phylogenetic position of the Remipedia is the subject of ongoing controversial debates. Some authors have interpreted the large number of similar trunk segments and the lack of a tagmatization of the trunk as ancestral features, indicating a basal position of the Remipedia within Crustacea (5, 6). Based on a comparison of the limb morphology and also on 18S-rRNA-encoding-DNA sequence data, others have advocated a close relationship to the Copepoda, a subgroup of the Maxillopoda (7, 8). A more recent molecular study analyzing elongation factor and RNA polymerase genes even suggested a position of the Remipedia outside of the Crustacea as the sister group of the Hexapoda (9). Interestingly, a rather similar hypothesis (a Remipedia–Malacostraca–Tracheata clade) had already been proposed, based on a compilation of morphological data (10). To sum up, the antipodes of this discussion range from an interpretation of the Remipedia as the most ancestral crustacean group to a position close to the Tracheata or Insecta.

Although the gross anatomy of some organ systems has already been analyzed in Remipedia (2–4, 11, 12), our knowledge about their anatomy is still very limited. Studies on the structure of the central nervous system of other crustaceans recently have provided a wealth of characters contributing valuable arguments to the debate on arthropod phylogeny (13–17). However, the brain architecture of Remipedia has not been explored so far. Therefore, we present a detailed histological study and reconstruction of the brain anatomy of *Godzilliognomus frondosus* Yager, 1989, (Remipedia, Godzilliidae) from the Grand Bahama Island (12) followed by a discussion of ecological and phylogenetic implications.

Methods

The cephalothorax of a formalin-fixed *Godzilliognomus frondosus* Yager, 1989, (Remipedia, Godzilliidae) specimen was embedded in Unicryl (British Biocell International, Cardiff, U.K.). Slices of 2.5 μm were sectioned by using a Reichert-Jung 2050-supercut. After toluidine-blue staining, digital images were taken by using a Microscope (Olympus BX40, Melville, NY) and a charge-coupled device camera (Olympus DP50). Three-dimensional reconstructions were done by using the surface-rendering software SURFDRIVER 3.5 (David Moody and Scott Lozanoff, www.surfdriver.com).

Results and Discussion

One outstanding feature of the anterior brain of *G. frondosus* is the inverted neuroaxis, caused by the striking elevation of the proto- and deutocerebrum, which additionally are bent almost 180° backwards so that the neuraxis is inverted with respect to the body axis. In consequence, the protocerebrum is oriented upside-down and located posteriorly to the deutocerebrum, which points upwards/backwards with the olfactory neuropils sticking out anteriorly (Fig. 1). Nevertheless, the ventral nerve cord and the tritocerebrum roughly are in line with the anterior–posterior body axis. Therefore, with the exception of Fig. 1, the architecture of the brain of *G. frondosus* will be discussed here with regard to the neuraxis only and not to the body axis (Fig. 2 *Inset*).

Blind but Worth Seeing: The Protocerebrum. Major components of the protocerebrum are the paired hemiellipsoid bodies (HEs), the olfactory-globular tracts (OGTs), and the central complex (Figs. 1 *d* and *e* and 2 *a–c*). The HEs are neuropils with a fine, dense texture (Fig. 2 *c* and *d*) that are linked to the olfactory neuropils by the OGTs (Fig. 1 *d* and *e* and twin asterisks in Fig. 2 *a*, *b*, and *d–h*; OGT in Fig. 3) as is the case in malacostracan crustaceans (18, 19). The two arms of this tract touch each other medially, forming a characteristic chiasm (Figs. 1, 2*e*, and 3), located next to the central body (CB, see below). As in

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Abbreviations: HE, hemiellipsoid body; CB, central body; OGT, olfactory-globular tract; PB, protocerebral bridge; A1Nv, nerves of the first antenna; ORN, olfactory receptor neurons; ON, olfactory neuropil; OS, olfactory satellite neuropil.

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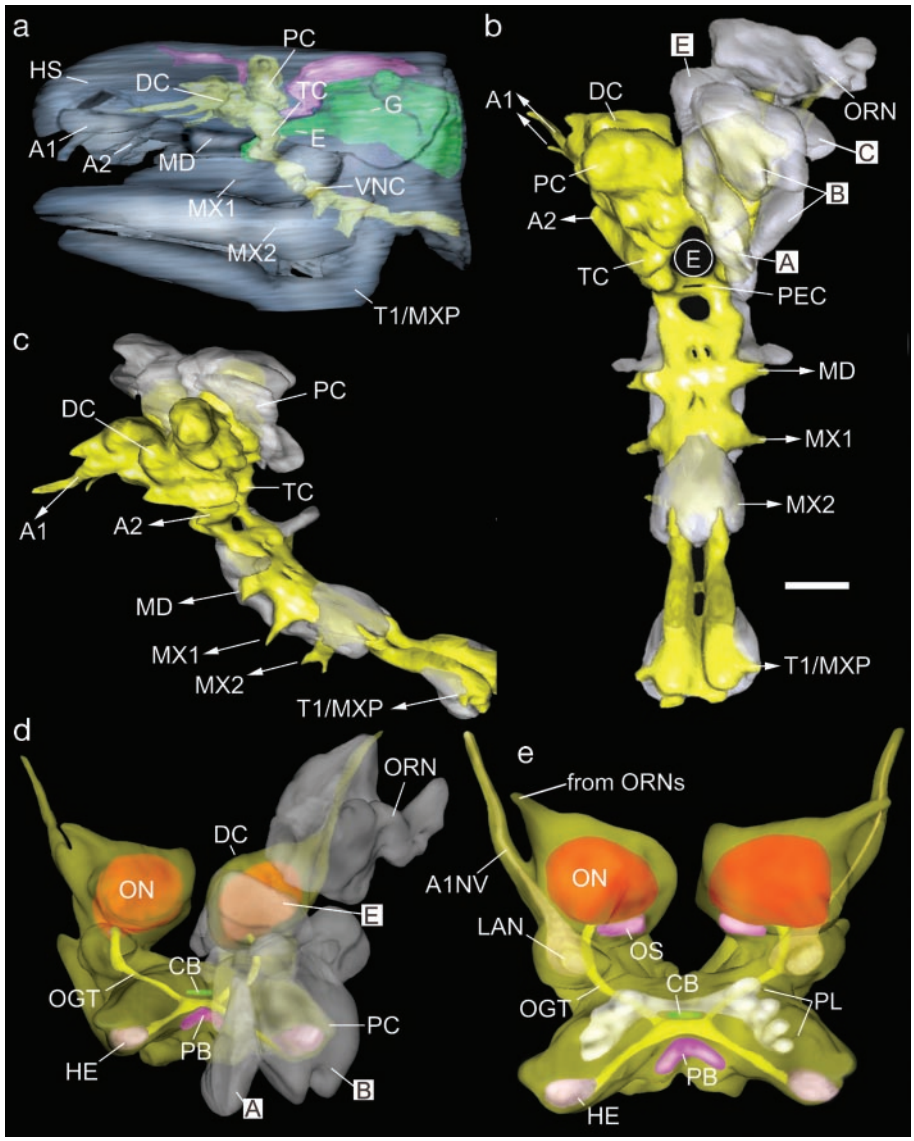

Fig. 1. Three-dimensional renderings of the anterior nervous system and some selected details of the proto (PC)- and deutocerebrum (DC) of the brain of *G. frondosus*. (*a*) Lateral view of the arrangement of the nervous system (yellow), gut (green), and heart (magenta) within the cephalothorax; anterior is toward the left. (*b*) Anterior nervous system seen from posteriodorsal with neuropil (yellow) and clusters of neuronal somata (gray) [A, B, and C of the olfactory receptor neurons (ORN)]; anterior is toward the top. (*c*) Same as *b* but in lateral view; anterior is to the left. (*d*) Rendering of PC and DC, showing the arrangement of olfactory neuropil (ON), olfactory globular tract (OGT), hemiellipsoid bodies (HE), central body (CB), protocerebral bridge (PB), and corresponding clusters of neuronal somata (A, B, and C, ORN); orientation is according to the body axis not neuraxis with anterior to the top. (*e*) Same as *d* but additionally showing the olfactory satellite neuropils (OS), the lateral antennal neuropils (LAN), A1Nv, and the protocerebral lobes (PL); orientation is as in *d*. A, B, and C, clusters of neuronal cell somata; A1, first antenna; A2, second antenna; DC, deutocerebrum; E, esophagus; G, gut; HS, head shield; MD, mandible; MX1, first maxilla; MX2, second maxilla; PEC, postesophageal commissure; T1/MXP, first thoracopod or maxilliped; TC, tritocerebrum; VNC, ventral nerve cord. (Scale bar: 200 μm.)

Malacostraca (20), the fibers in the remipede OGTs seem to partly cross contralaterally, whereas others pass ipsilaterally (arrows in Fig. 2*e*). The protocerebrum is subdivided into at least four sublobes in addition to the HE (Fig. 3, A–D). Within both hemispheres, lobe a is located next to the HE (Fig. 3). Therefore, these structures together may constitute the medulla terminalis (compare refs. 18–20). The central complex in crustacean brains [and, most likely, also the mandibulate ground pattern (14, 16, 17)] is composed of the CB, an unpaired midline neuropil, which is accompanied by several commissural fiber bundles, the lateral lobes, and the protocerebral bridge (PB) (14, 16, 21). These components are also recognizable in the brain of *G. frondosus*, although the CB is rather small (Figs. 1 *d* and *e* and 2 *a*, *b*, and *f*), perhaps because of the absent visual input. Based on the location of the commissural fiber bundles, we suggest that the paired protocerebral lobes d (Fig. 2*b*), each of which seems to be subdivided into three units (d1–d3, Fig. 2*a*), correspond to the lateral lobes known from other crustaceans (16, 21). Three massive paired clusters of neuronal somata, labeled A, B, and G in Fig. 2 *a–c* and *f*, are associated with the protocerebrum. Dendrites from these neurons invade the protocerebral neuropil (arrow in Fig. 2*a*). The neuronal somata in Malacostraca are also clustered in characteristic groups (18, 19). We propose that the remipede clusters A/B and G correspond to the malacostracan clusters 6/7 and 12/13, respectively.

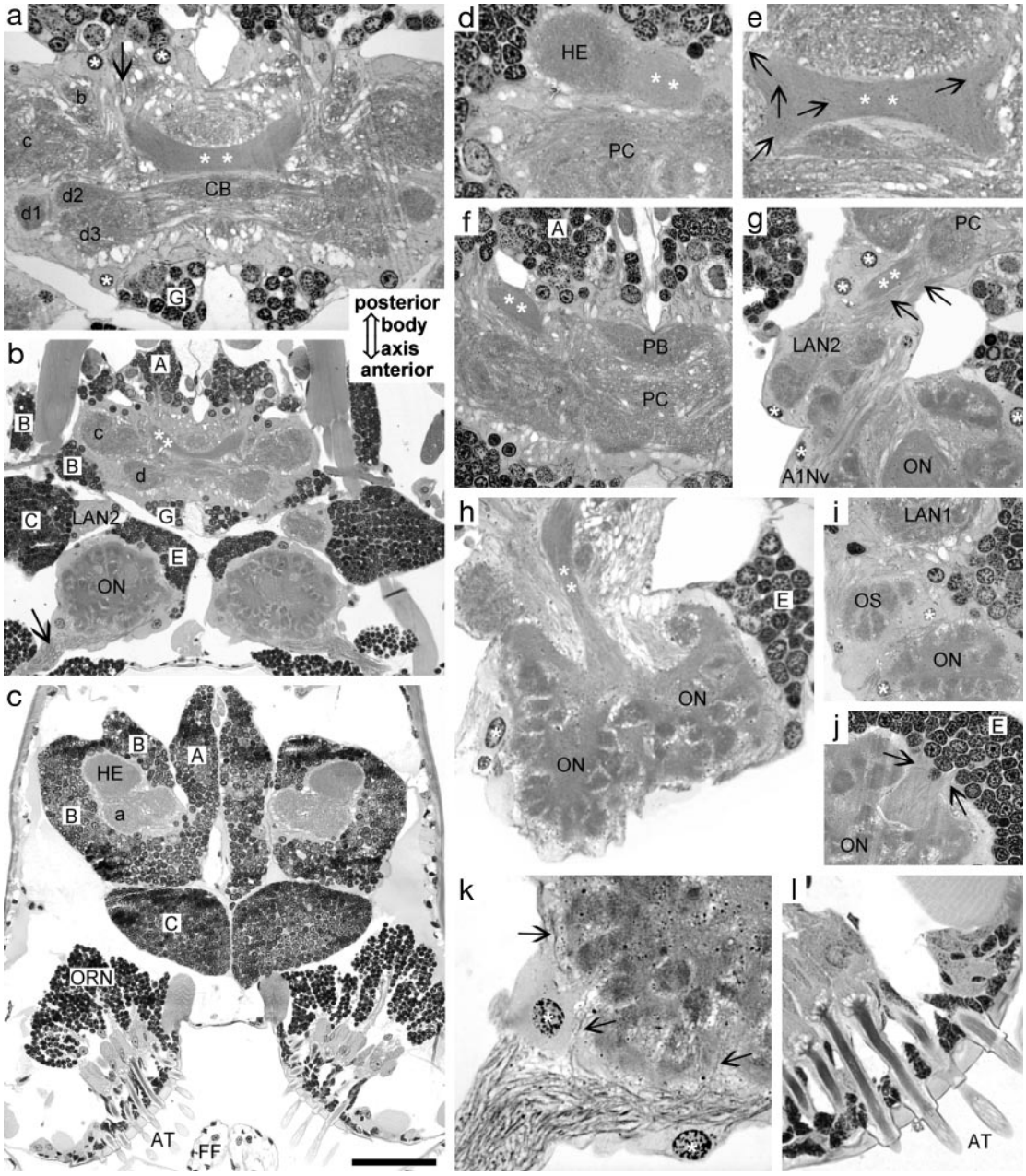

Fig. 2. Horizontal brain sections. The anterior of the neuraxis is toward the top, and the orientation of the body axis is indicated in the *Inset*. Single asterisks in *a*, *g–i*, and *k* identify the nuclei of putative glial cells. Twin asterisks identify the OGT. Capital letters in boxes label clusters of neuronal cell somata. (*a*) Protocerebrum with components of the central complex. The arrow identifies fiber bundles that emerge from cell cluster A and invade the protocerebrum. (*b*) Low magnification; arrow identifies axons of ORNs that invade the ON. (*c*) Low magnification somewhat dorsal to *b*, showing clusters of ORNs associated with aesthetascs (AT). (*d*) The OGT (double asterisks) enters the HE. (*e*) Chiasm of the bilateral OGTs. Arrows identify the putative course of axons within the chiasm. (*f*) Protocerebrum (PC) with PB. (*g*) The lateral antenna 1 neuropil 2 (LAN2) is innervated by the A1Nv, asterisks identify fibers from the LAN2 that proceed toward the PC. (*h*) The OGT (twin asterisks) as it exits the ON. (*i*) The OS. (*j*) Fiber bundles from cell cluster E (asterisks) invade the ON. (*k*) Axons of ORNs invade the ON (arrows). Asterisks identify the nuclei of putative glial cells surrounding the ON. (*l*) Higher magnification of the AT. a–c and d1–d3, sublobes of the protocerebrum; A2Nv, antenna 2 nerve; CO, connective; E, esophagus; EG, esophageal ganglion; FC, frontal commissure; LAN1 and -2, lateral antenna neuropils 1 and 2; MAN, median antenna 1 neuropil; PEC, postesophageal commissure; TC, tritocerebrum; TNV, tegumentary nerve. (Scale bar in *c*: 200 μm.)

The Deutocerebrum: Sophisticated Olfactory Processing. The deutocerebrum adjoins the protocerebrum rostrally (Fig. 1). It consists of the median antenna 1 neuropil (MAN) (Fig. 3), a relatively diffuse block of neuropil crossing the midline of the brain, which contains a transverse commissural fiber bundle linking the deutocerebral hemispheres. Furthermore, the paired lateral antenna 1 neuropils [LAN in accordance with the malacostracan nomenclature (18, 19)] are part of the deutocerebrum and receive a distinct input from the nerves of the first antennae (A1Nv). They are associated with cell clusters C and D (Figs. 1 and 3). On both sides, the LAN is subdivided into two distinct compartments (LAN1 and LAN2; Fig. 2 *b*, *g*, and *i*), as in malacostracan crustaceans (18). The A1Nv most likely are mixed sensory and motor nerves and innervate both rami of the first

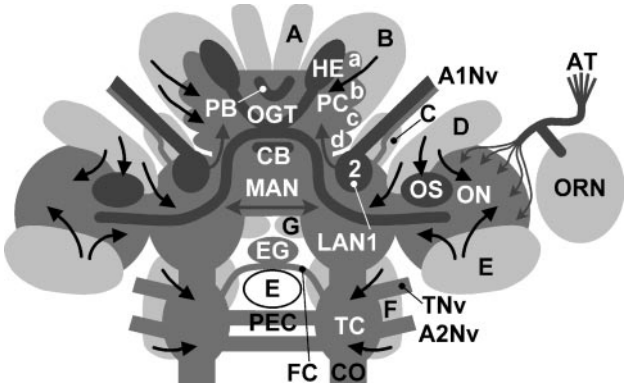

Fig. 3. Two-dimensional schematic representation of the brain, straightened out according to the neuraxis, ONs turned out laterally. A–G label identified cell clusters. Arrows show fibers from these clusters that invade the neuropil. For other abbreviations, see the legend to Fig. 2.

antennae, which are equipped with numerous setae (12). As they approach the brain, the A1Nv split up into a smaller and a larger portion, which target the LAN1 and LAN2, respectively (Figs. 2*g* and 3). This separation may coincide with a separation of motor and sensory qualities within the nerves. In malacostracans, the LAN receives afferents from mechanoreceptors and nonaesthetasc chemoreceptors (18). In *G. frondosus*, the LAN2s are subdivided into several distinct glomeruli (Fig. 2*g*) and are associated with distinct cell clusters with hundreds of neuronal somata (cluster C, Figs. 2*c* and 3). We therefore suggest that the LAN2s process mechano- and/or chemosensory input rather than being motor neuropils. Fibers emerging from these structures project anteriorly (arrows in Fig. 2*g*) in parallel with the OGT and enter the protocerebrum.

The basal segments of the antennae 1 in remipedes 2–4 are equipped with dense tufts of olfactory receptors, the aesthetascs (AT, Fig. 2*c*). These are arranged in several rows and in *G. frondosus* amount to ≈40 on each side. The somata of the olfactory receptor neurons (ORNs) are arranged in conspicuous clusters within the basal portion of the first antennae immediately adjacent to the brain (Figs. 1 and 2*c*). These clusters are composed of thousands of somata and provide a massive input to yet another pair of deutocerebral neuropils, the dominating olfactory neuropils (ONs; Figs. 1, 2 *b* and *g–k*, and 3). The massive fiber bundles from the ORN clusters approach the ONs from a ventral/anterior direction, then split up into numerous branches that spread around the spherical ONs and penetrate into the neuropil (arrows in Fig. 2*k*) much as in Malacostraca (22). The neuropil of the ONs is differentiated into dozens of characteristic olfactory glomeruli (Fig. 2 *b*, *h*, *i*, and *k*), which give it the shape of a cauliflower (Fig. 2*h*). In Malacostraca, in which these glomeruli have a slightly different architecture, they serve as functional units for olfactory processing and are the sites where the primary chemosensory afferents contact the dendrites of second-order neurons (22–25). A small additional neuropil, the olfactory satellite neuropil (OS), is located between the ON and LAN1 on both sides of the remipede brain (Fig. 2*i*). Two paired cell clusters, D and E, both of which comprise several hundreds of neuronal somata, are associated with each ON. Fibers emerging from these clusters target the core of the neuropil (Fig. 2*j*), suggesting that these neurons are olfactory interneurons, an arrangement that closely resembles that in Malacostraca (22–25). We suggest that the remipede clusters D and E correspond to the malacostracan clusters (18) 9/11 and 10, respectively. The OGTs in the remipede brain appear as thick fiber tracts that emerge from the ONs to veer anteriorly (Fig. 2*h*) and are composed of the axons of olfactory projection neurons that target the HEs, as in Malacostraca (20).

The Third Unit of the Brain. The tritocerebrum adjoins the deutocerebrum ventrally (Fig. 1). It is associated with the antenna 2 nerves and the tegumentary nerves innervating the integument of the cephalic shield (Figs. 1 and 3). The paired tritocerebral lobes are transversely joined by a double postesophageal commissure. They also give rise to a frontal connective (Fig. 3), which innervates a first unpaired frontal ganglion rostral to the esophagus, from which a nerve projects ventrally to innervate the labrum (data not shown). The first frontal ganglion also is connected to a second unpaired frontal ganglion, the esophageal ganglion (Fig. 3), which is located more dorsally than the first one. The tritocerebrum is associated with cell cluster F, which may correspond to the malacostracan clusters 14, 15, or 16 (18). The tritocerebrum is adjoined by the subesophageal mass corresponding to the mandibular, the two maxillar, and the maxilliped segments (Fig. 1 *b* and *c*). The subesophageal mass is followed by a ventral nerve chain with paired ganglia, connectives, and commissures within each trunk segment (data not shown).

Brain, Behavior, and Ecology. Clearly, in the absence of any visual input to the protocerebrum, the deutocerebrum acts as the dominating part of the remipede brain. The gigantic olfactory apparatus of *G. frondosus* suggests that in the aphotic cave habitat chemical cues are a major source of sensory information for these animals. With their second antennae continuously beating to generate a water current across the fields of aesthetascs on the first antennae (3, 4) and thousands of olfactory receptor neurons and olfactory interneurons ready to process this chemosensory input, we have to expect that these animals can detect and orient toward extremely low odor concentrations from potential food items. This claim is supported by the observation that in their habitat, remipedes are attracted by bait of partially decomposed fish within a few minutes, suggesting that they likely feed on carrion washed into the cave or on any fallout of dead animals from upper water levels of the cave (4). Evidence obtained during cave dives suggests that that they also feed on dead blind cave fish (J. Yager, personal communication). Remipedia share their habitat with other crustaceans such as ostracods and a variety of malacostracans (1–4, 12) and they are well equipped for predation with numerous mechanosensors, fang-like mouthparts, and a pair of voluminous poison glands discharging on the tip of the first maxilla. Observations during cave dives and of animals kept in aquaria recently provided evidence that remipedes in fact actively prey on blind cave shrimps (J. Yager, personal communication) as had been suggested earlier (26). This evidence is remarkable, because a recent analysis of locomotion in Remipedia revealed that they move relatively slowly and their mode of swimming seems optimized for saving energy in an environment that is poor in oxygen and food (27). In conclusion, predation on other cave crustaceans and scavenging may play equally important roles in their feeding strategy.

Phylogenetic Implications: A Remipedia–Malacostraca–Hexapoda Clade? The arrangement of nerves, axonal tracts, neuropil compartments and cell clusters in the brain of *G. frondosus* resembles that of Malacostraca more than that of any other crustaceans. Nevertheless, to find out whether these features are plesiomorphic characters (ancestral characters shared by other arthropods as well), a comparison with other groups is necessary. Detailed information on the brain anatomy that allows such a meaningful comparison is available for hexapods (e.g., ref. 14), as well as for representatives of the Branchiopoda (15, 16, 28–30) and Max-

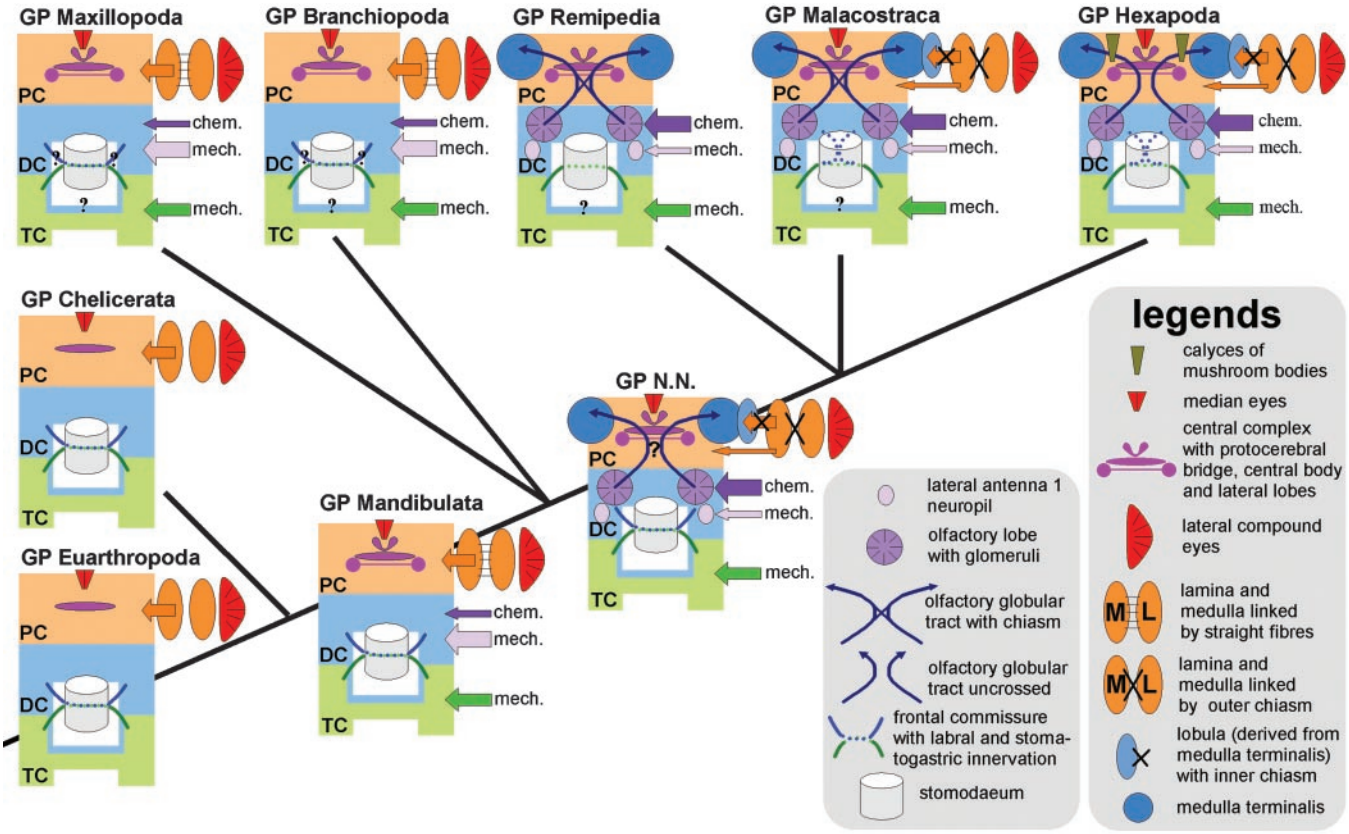

Fig. 4. An analysis of phylogenetic relationships and brain morphology in the ground patterns (GP) of the various taxa discussed in this article. This analysis is founded exclusively on brain anatomy, deliberately ignores all other morphological characters as well as fossil taxa, and excludes the ''Myriapoda'' because of lack of data. The correspondence of the three brain neuromeres of Chelicerata and Mandibulata has recently been demonstrated (36, 45), and a homology of chelicerate and mandibulate central bodies has been suggested (46). In the Mandibulata, arrows indicate a predominantly chemosensory (chem.) or mechanosensory (mech.) input to the brain. Recent neuroembryological studies on representatives of the Chelicerata and Mandibulata (36, 45) have shown that the postoral commissure has both deuto- and tritocerebral components, thereby providing evidence that, in the euarthropodan brain, the stomodaeum is not located between the deuto- and the tritocerebrum but within the deutocerebrum. Likewise, the frontal commissure that gives rise to the labral and stomatogastric innervation has both deuto- and tritocerebral components (36). The mushroom bodies in our view are an apomorphy of the Hexapoda and are not related to structures termed ''mushroom bodies'' in other Arthropoda or the Annelida (14).

illopoda (28, 31, 32) (Fig. 4). Specifically, the brains of branchiopods and maxillopods typically are covered by only a thin cortex (≈2–4 cell layers) of neuronal somata, unlike the remipedes, malacostracans, and hexapods, which have clusters with thousands of neurons. We estimate that the number of neurons in the remipede brain exceeds that in Branchiopoda and Maxillopoda at least by one order of magnitude. The brains of these ''lower'' crustaceans are far less differentiated and complex than the remipede brain. Concerning neuropil structures, the central complex most likely is a plesiomorphic feature taken over from the mandibulatan ground pattern, present in Malacostraca as well as in Branchiopoda, Maxillopoda, Hexapoda, and Chilopoda (14, 16, 17, 21, 28). However, structures such as glomerular ONs (which are distinctly set apart from the remaining deutocerebrum), bipartite antenna 1 neuropils, OGTs with a characteristic chiasm, and the HEs (as part of the medullae terminales) are characters that are absent in the Branchiopoda and Maxillopoda and shared only by Remipedia, Malacostraca, and, with slight differences [olfactory globular tracts without chiasm and differences in the organization of the olfactory system (14)], also by hexapods (Fig. 4).

There are two models to explain these striking similarities among Remipedia, Malacostraca, and Hexapoda. (*i*) The brain was already ''complex'' in the mandibulatan ground pattern, and consequently the maxillopodan and branchiopodan brains have been strongly simplified. This explanation implies that the similarities mentioned are symplesiomorphies (shared ancestral characters) that do not support a close phylogenetic relationship of Remipedia, Malacostraca, and Hexapoda. (*ii*) Alternatively, we can assume a ''simple'' situation in the mandibulatan ground pattern like that seen in Branchiopoda and Maxillopoda, followed by the evolution of a more complex brain. Because the tritocerebral neuromere in Branchiopoda and Maxillopoda is in a postoral position, their brains have been suggested to display many plesiomorphic features (33). Moreover, an outgroup comparison shows that the specific characters mentioned above are also absent in Chelicerata (34–36), and therefore we conclude that they most likely were not present in the mandibulatan ground pattern. Hence, we propose that olfactory lobes with glomeruli, bipartite antenna 1 neuropils, the OGTs, and a medulla terminalis with HEs (the latter correspond to the lateral horn in the lateral protocerebrum in Hexapoda; Fig. 4) are synapomorphies that unite Remipedia, Malacostraca, and Hexapoda. A further synapomorphy of these three groups is the presence of a third optic neuropil (lobula), as well as an inner and outer optic chiasm (15). We assume these structures to be lost in the remipede brain because of the remipede's total absence of eyes (Fig. 4). In conclusion, our study does not provide arguments in favor of the hypothesis that the Remipedia may be the most ancestral

crustacean group (5, 6) and also casts doubt on a close affinity to the Copepoda (7, 8). Because the chiasm of the OGTs is present only in Remipedia and Malacostraca but absent in insects, this character argues in favor of a sister-group relationship of Remipedia and Malacostraca.

Problematic Tracheata. Molecular studies have questioned the traditional understanding of arthropod relationships, frequently refuting the monophyly of the Tracheata and instead supporting a sister-group relationship of Hexapoda and Crustacea (e.g., refs. 9 and 37). The morphological characters that support tracheatan monophyly (e.g., refs. 38 and 39) have been questioned by other authors (e.g., refs. 40 and 41). Moreover, studies on brain anatomy (13–17) and on the structure and development of the ventral nerve cord (42, 43) have revealed striking similarities of malacostracan and hexapodan nervous systems. Because of the lack of data concerning the "myriapodan" taxa, we have included only the Hexapoda in our phylogenetic analysis (Fig. 4), leaving the matter of tracheatan monophyly unconsidered so far. If monophyletic, the most parsimonious placement of the Tracheata would be within a clade Remipedia + Malacostraca + Tracheata (instead of Hexapoda in Fig. 4). We have to emphasize that this analysis is founded exclusively on brain anatomy, deliberately ignoring all other morphological characters, as well as fossil taxa. Nevertheless, our hypothesis of a Remipedia–Malacostraca–Hexapoda clade (taxon N.N. in Fig. 4) is also in line with some molecular (9, 37) and morphological (10, 40, 41) data but clearly contradicts our traditional understanding of arthropod phylogeny (38, 39, 44).

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